Research article

Neuroprotective effects of topiramate and memantine in combination with hypothermia in hypoxic-ischemic brain injury in vitro and in vivo

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1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a major cause of perinatal mortality and subsequent severe neurological sequelae. Mild hypothermia is a standard therapy for HIE, but is used only in selected Reference Centers and in neonates > 1800 g. Since neuronal death following HIE occurs by a cascade of events triggered by activation of glutamate receptors, we used in vitro and in vivo models of HIE to examine whether the AMPA/kainate receptor antagonist topiramate and the NMDA receptor antagonist memantine could exert neuroprotective effects, alone or in combination with hypothermia. For the in vitro experiments, rat organotypic hippocampal slices were exposed to a 30 min duration of oxygen-glucose deprivation (OGD): treatment with topiramate (1 μM) and memantine (10-30 μM) or hypothermia (35 °C or 32 °C) significantly attenuated CA1 damage after 24 h. The combination of hypothermia with topiramate and memantine enhanced their protective effect. For the in vivo experiments, we used 7 day-old rat pups subjected to permanent left common carotid artery occlusion followed by 120 min of hypoxia. Administration of topiramate or memantine (i.p., 20 mg/kg) immediately and 2 h after hypoxia or exposure to hypothermia (32 °C for 4 h beginning 1 h after hypoxia) significantly reduced the extent of the resulting infarct. The combination of topiramate or memantine with hypothermia elicited a reduction of the infarct that was greater than that produced by drugs or hypothermia alone. Notably, memantine displayed a higher degree of neuroprotection as compared to topiramate both in vitro and in vivo and, when used alone at 20 mg/kg in vivo, produced a greater reduction in brain damage than observed using topiramate in combination with hypothermia. These results suggest that memantine may be more advantageous than topiramate as a therapeutic agent in neonates with HIE treated with hypothermia.

A R T I C L E  I N F O

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A B S T R A C T

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neuronal damage in experimental models of HIE. Topiramate has also been shown to extend the therapeutic window for neuroprotection induced by hypothermia in hypoxic-ischemic neonatal brain injury [10].

Recently, we reported that topiramate in neonates with HIE treated with hypothermia (NeoNATI study) is safe, does not reduce the combined frequency of mortality and severe neurological disabilities but may reduce the prevalence of epilepsy [6].

In the present study, we used in vitro and in vivo models of HIE to examine whether memantine as compared to topiramate could exert neuroprotective effects alone or in combination with hypothermia.

2. Materials and methods

2.1. Animals

Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The experimental protocols were approved by the Animal Care Committee of the Department of Health Sciences, University of Florence, in compliance with the European Convention for the Promotion of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.2. Materials

Topiramate and memantine hydrochloride were purchased from Tocris (Avonmouth, Bristol, UK). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

2.3. Oxygen-glucose deprivation in organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported [11]. Briefly, hippocampi were removed from the brains of 7–9 days old Wistar rats (Harlan, MI, Italy), and transverse slices (420 µm) were prepared using a McIlwain tissue chopper. Slices were then transferred onto 30 mm diameter semiporous membranes inserts (420 µm) were prepared using a McIlwain tissue chopper. Slices were then transferred onto 30 mm diameter semiporous membranes inserts. Slices were maintained at 37 °C in an incubator in atmosphere of humidified tissue culture plates containing 1.2 ml culture medium per well. Then transferred onto 30 mm diameter semiporous membranes inserts (420 µm) were prepared using a McIlwain tissue chopper. Slices were then transferred onto 30 mm diameter semiporous membranes inserts. Slices were maintained at 37 °C in an incubator in atmosphere of humidified air and 5% CO₂ and the culture medium was changed thrice a week. Experiments were carried out after 14 days in vitro. The slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N₂ and 5% CO₂ at 37 °C in an airtight anoxic chamber equipped with an oxygen gas controller (BioSpherix, New York, USA). After 30 min, the cultures were transferred to oxygenated serum-free medium containing 5 mg/ml glucose and maintained at 37 °C, 35 °C or 32 °C in different incubators under normoxic conditions until neuronal injury was evaluated 24 h later. Topiramate and memantine were present in the incubation medium during OGD and the subsequent 24 h recovery period. Cell injury was assessed by using the fluorescent dye propidium iodide (5 µg/ml); fluorescence was viewed using an inverted fluorescence microscope. Images were digitized using a video image controlled by software and subsequently analyzed using a morphometric analysis software. In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected.

2.4. Neonate rat model of hypoxic-ischemic encephalopathy

Surgical procedures were performed on postnatal day 7 Wistar rats (Harlan, MI, Italy), following the Rice-Vannucci model described by [12]. Briefly, rat pups were anesthetized with isoflurane gas (induction, 4% maintenance, 1%) in oxygen:nitrous oxide (1:1). The left common carotid artery was ligated using 4-0 silk following a midline incision. After the operation, all pups were returned to their dams for recovery and lactation for 1.5 h. Then pups were placed in enclosed, vented Plexiglas chambers (W20, D20, H17) partially submerged in a water-bath and exposed by continuous flow to warmed, humidified gas 8% oxygen 92% nitrogen for 120 min. After the insult, the pups returned to their dams for 1 h, and then they were placed in a chamber at normothermic (37 °C) or hypothermic (32 °C) temperatures for 4 h. Topiramate and memantine were administered i.p. immediately and 2 h after hypoxia, according to the regimen proposed for topiramate by Noh et al. [8] and at doses that were shown to be stable and safe following hypothermia in infants [13]. All pups were then maintained with their dam and sacrificed by decapitation under isoflurane anesthesia 72 h later. For the measurement of the infarct areas and volumes, 1 mm thick coronal sections were cut with a blade, incubated in 2% 2,3,5- triphenyl-tetrazolium chloride (TTC) for 20 min at 37 °C, and then fixed in 4% paraformaldehyde for 24 h. Infarct areas were measured using a computerized image analysis system (Image-Pro Plus 3.0, Silver Spring, MD, U.S.A.). The infarct volume was calculated as described [14].

2.5. Statistical analysis

Data are presented as means ± SEM of n experiments. Statistical significance of differences between PI fluorescence intensities or TTC staining was analyzed using one-way ANOVA followed by the post hoc Dunnet and Tukey’s w-test for multiple comparisons. All statistical calculations were performed using GRAPHPAD PRISM v. 5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (P) of < 0.05 was considered significant.

3. Results

3.1. Neuroprotective effects of topiramate and memantine alone or in combination with hypothermia in rat organotypic hippocampal slices exposed to OGD in vitro

To investigate the neuroprotective effects of topiramate and memantine we exposed rat organotypic hippocampal slices to 30 min OGD, which produces selective CA1 injury 24 h later [11]. Hippocampal slices exposed to topiramate or memantine alone for 24 h displayed no apparent signs of neurodegeneration (data not shown). Fig. 1A shows that topiramate (0.01–1 µM) and memantine (1–30 µM) produced a concentration-dependent neuroprotection when present in the incubation medium during OGD and the subsequent 24 h recovery period. Similarly, when slices were maintained under hypothermic conditions during the subsequent 24 h after OGD, we observed that reducing the temperature to both 35 °C or 32 °C attenuated neuronal damage in the CA1 region by approximately 35% and 55%, respectively (Fig. 1B). The combination of 32 °C hypothermia with topiramate elicited a potentiation of its protective effect, that was significant at 0.1 µM topiramate (Fig. 1C). The addition of 30 µM memantine to the medium in combination with 35 °C hypothermia showed a neuroprotective effect that was greater than 35 °C hypothermia alone (Fig. 1D), whereas memantine (30 µM) in association with 32 °C hypothermia displayed a reduction of neuronal death that was greater than both deep hypothermia alone and 30 µM memantine at 37 °C (Fig. 1D).

3.2. Neuroprotective effects of topiramate and memantine alone or in combination with hypothermia in the neonate rat model of hypoxic-ischemic encephalopathy

In accordance with Rice et al. [21], left carotid artery occlusion followed by exposure to hypoxia (8% oxygen 92% nitrogen for 2 h)
resulted in a 80% incidence of ipsilateral cerebral infarction in P 7 vehicle-treated rat pups. Histopathologic findings on the hemisphere ipsilateral to the carotid artery occlusion included pallor, atrophy, and tissue loss in the striatum, hippocampus, cortex and thalamus (data not shown). When brain slices were stained with TTC, we observed that HIE produced a dramatic brain infarct (Fig. 2A) that was quantified in 64.9 ± 5 mm³ (mean ± SEM). Administration of topiramate or memantine i.p. immediately and 2 h after hypoxia significantly reduced the extent of the infarct in a dose-dependent manner, reaching a 49 ± 15% and 78 ± 5% reduction, respectively, at 20 mg/kg, whereas hypothermia (32 °C for 4 h beginning 1 h after the end of hypoxia) significantly reduced the extension by approximately 55 ± 4% (Fig. 2B). In corroboration of previous studies [8], topiramate and memantine displayed similar neuroprotective effects both in male and female rats (data not shown). According to previous Topiramate The combination of topiramate with hypothermia elicited a reduction of the infarct that was greater than that produced by topiramate alone, whereas the combination of memantine with hypothermia elicited a reduction of the infarct that was greater than that produced by both memantine and hypothermia alone (Fig. 2B).

4. Discussion

In this study, we used in vitro and in vivo experimental models of hypoxic-ischemic brain injury to explore the hypothesis that memantine, as well as topiramate, may be used as a neuroprotective agent in neonates with HIE treated or not with hypothermia. Our results show that under our experimental conditions both topiramate and memantine reduce neuronal damage following hypoxia-ischemia in brain neonatal tissue, and that their protective effects are enhanced when they are used in combination with hypothermia.

Therapeutic hypothermia is the standard of care for improving survival and reducing the neurodevelopmental sequelae in neonates with moderate to severe HIE. Although relatively few preclinical studies have compared multiple hypothermic treatment temperatures, it has been recently proposed that, since cooling below 33.5 °C does not appear to provide additional neuroprotection in neonatal rat models of HIE, relatively mild cooling should be considered for future clinical trials [15]. Hypothermia is indeed effective and has not been associated with major complications, but appears to be beneficial only to a relatively small percentage of infants with HIE [16]. Specifically, treatment with therapeutic hypothermia is currently reserved to newborns born at term or near term (> 1800 g), and is usually restricted to Reference Centers with specific expertise and equipment. Therefore, neuroprotective drugs that are able to enhance the effects of mild hypothermia may not only be an extremely valuable addition to current clinical practice but may represent the treatment of choice for preterm newborns or neonates born in areas where cooling is not possible or available.

Our results show that exposure of neonatal brain tissue to hypothermia, both in vitro and in vivo, produced a marked attenuation of brain damage following hypoxic-ischemic injury. Under our in vitro
Fig. 2. Topiramate and memantine alone or in combination with hypothermia are neuroprotective in a neonate rat model of hypoxic-ischemic encephalopathy. P7 rat pups were subjected to left carotid artery occlusion followed by exposure to hypoxia. Topiramate or memantine were injected i.p. immediately (0 h) and 2 h after the end of hypoxia. Hypothermia (32 °C for 4 h) was applied starting 1 h after the end of hypoxia. (A) Top: Diagram of the experimental protocol. Bottom: Representative coronal brain sections stained with TTC. The infarct area appears white. HIE induced a dramatic cortico-striatal lesion that was reduced by 20 mg/kg topiramate or memantine. (B) Quantitative analysis showing that topiramate (10–20 mg/Kg), memantine (5–20 mg/Kg) and hypothermia (32 °C) significantly reduced the infarct volume induced by HIE. Topiramate plus hypothermia displayed a greater neuroprotective effect than topiramate alone, memantine plus hypothermia showed greater neuroprotection than memantine or hypothermia alone. Bars represent the mean ± SEM of at least 6 animals. *P < 0.05 and **P < 0.01 vs. HIE at 37 °C, @P < 0.05 vs. HIE + 10 mg/Kg topiramate at 37 °C, #P < 0.05 vs. HIE + 5 mg/Kg memantine at 37 °C and HIE at 32 °C (ANOVA + Tukey’s w test in B).
conditions, slices exposed to 32 °C displayed a reduction of CA1 pyramidal cell death that was significantly more robust than that observed in slices exposed to 35 °C. When neuroprotective agents were tested by themselves, topiramate and memantine significantly attenuated neuronal damage in our models. The doses and the administration protocols of drugs were selected in order to achieve a potential clinical application, and were extrapolated from both their IC50 (0.46 μM for kainate receptors in the case of topiramate and 1.25 μM for NMDA receptors in the case of memantine) and their previous in vitro, in vivo and clinical use, showing that doses up to 20 mg/kg are both safe and effective. The maximal attenuation of neuronal damage in vitro was observed at 1 μM topiramate and 30 μM memantine; higher concentrations did not provide an increased neuroprotection but rather produced bell-shaped curves with a loss of the effect (not shown), possibly due to non-specific effects of the drugs on other targets. On the other hand, in the neonate rat model of HIE in vivo, both drugs displayed their maximal degree of protection at the same dose of 20 mg/kg. Hypothermia at 32 °C further enhanced the neuroprotective effects of relatively high concentrations of topiramate and memantine in vitro, but was able to markedly potentiate the effects of the lowest doses of both drugs in vivo. Although the comparison did not reach statistical significance, memantine exhibited a trend towards a higher degree of neuroprotection as compared to topiramate both in vitro and in vivo, and when used alone at 20 mg/kg in vivo produced a trend towards a greater reduction in brain damage than that observed using topiramate in combination with hypothermia. These results suggest that memantine may be more advantageous than topiramate as a therapeutic agent in neonates with HIE treated with hypothermia.

In a recent pilot study, we evaluated the safety and efficacy of topiramate in add-on to moderate hypothermia in 44 newborns with HIE [6] and found that the treatment was safe, but observed no statistically or clinically significant differences for the combined frequency of mortality and severe neurological disability. Interestingly, there was a reduction in the prevalence of epilepsy in newborns co-treated with topiramate, suggesting that the negative results observed for the other primary or secondary outcomes could be due to the small sample size and hence larger clinical trials are needed. The results of the present study suggest that it would be appropriate to explore not only the efficacy of topiramate but also of memantine in neonates with HIE treated with hypothermia.

Memantine is very well tolerated when used to treat moderate-to-severe Alzheimer's disease in adults, likely due to its low affinity for NMDA receptor channels and its fast unblocking kinetics, and has also been reported to be relatively safe in the developing rat brain [17]. In the recent past, preclinical studies using in vitro and in vivo models of HIE have provided useful suggestions for novel therapeutic approaches to be used in Neonatal Intensive Care Units. This study shows that memantine provides a remarkable degree of neuroprotection, alone and in combination with hypothermia, which tends to be superior to that observed with topiramate under the same conditions. These data need to be supported by further studies with evaluation of functional outcomes, but may provide a proof of concept that clinical trials using memantine in neonates with HIE treated with hypothermia could be performed.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References